PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 13/00, A61K 35/28 C12N 15/19, A61K 37/02	**A1**	**(11) International Publication Number:** WO 91/04274 **(43) International Publication Date:** 4 April 1991 (04.04.91)

(21) International Application Number: PCT/US90/05451

(22) International Filing Date: 25 September 1990 (25.09.90)

(30) Priority data:
412,303   25 September 1989 (25.09.89)   US
581,713   13 September 1990 (13.09.90)   US

(71) Applicants: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). CRC TECHNOLOGY LTD. [GB/GB]; 2 Carlton House Terrace, London SW1Y 5AR (GB).

(72) Inventors: PRAGNELL, Ian, B. ; 14 Belmont Crescent, Glasgow G12 (GB). DONALDSON, Debra, D. ; 40 1/2 Roberts Road, Cambridge, MA 02138 (US). GRAHAM, Gerald, J. ; 31 Edgemont Street, Glasgow G41 3EH (GB). WONG, Gordon, G. ; 40 Jamaica Way, Apt. 10, Jamaica Plain, MA 02130 (US).

(74) Agents: KAPINOS, Ellen, J. et al.; Genetics Institute, Inc., 87 Cambridge Park Drive, Cambridge, MA 02140 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR INHIBITING GROWTH OF STEM CELLS

(57) Abstract

The present invention discloses a method for protecting cycling or dividing stem cells from the cytotoxic effects of chemotherapeutic agents and radiation by administering prior to exposure to these agents, an effective amount of Stem Cell Inhibitory Factor as well as a composition useful therefor.

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	PL	Poland
CA	Canada	JP	Japan	RO	Romania
CF	Central African Republic	KP	Democratic People's Republic	SD	Sudan
CG	Congo		of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
DE	Germany	LK	Sri Lanka	TD	Chad
DK	Denmark	LU	Luxembourg	TG	Togo
				US	United States of America

1

METHOD FOR INHIBITING GROWTH OF STEM CELLS

The present invention relates generally to the
treatment of human or animal subjects anticipating

5 exposure to chemotherapeutic agents, radiation exposure,
or other agents which irreparably damage cycling stem
cells and a stem cell inhibitory composition useful
therefore.

Cross-Reference to Related Application

10 This application is a continuation-in-part of
U. S. Patent Application, Serial No. 07/412,303, filed
September 25, 1989.

Background of the Invention

In the treatment of subjects with cancer,
15 conventional chemotherapy involves the application of one
or more cell cycle specific cytotoxic agents, which have
the adverse effect of killing, or irreparably damaging,

20

25

2

both normal and cancer cells undergoing division. The

goal of such chemotherapeutic agents, e.g., cytosine

arabinoside (ara-C), is to destroy or disable the

dividing cancer cells, preventing further cancer cell

5   growth. A presently unavoidable effect of chemotherapy,

however, is the destruction of other normal dividing

cells, particularly the stem cells of the hematopoietic

system and the epithelial stem cells which line the scalp

and gut. Stem cell damage caused by the administration

10   of chemotherapy, result in the usual side effects

thereof, such as hair loss, stomach and intestinal

damage, skin damage, myelosuppression, anemia, reduced

immune function or response and the resulting increased

sensitivity to infection.

15   A characteristic property of stem cells is

their quiescent nature within the cell cycle. When there

is 'insult' to the bone marrow, such as by drug

treatment, radiation, severe blood loss, inflammatory

reaction, or infection, the stem cell responds by a

20   feedback mechanism to begin cycling to replenish the more

mature progenitor cells, which in turn, differentiate

into the required mature cells of the hematopoietic,

immune or epithelial systems.

        Since the hematopoietic stem cells are

25   necessary for the development of all of the mature cells

of the hematopoietic and immune systems, their survival

is essential in order to reestablish in the subject

3

treated with chemotherapy, a fully functional host

defense system. Similarly, survival of epithelial stem

cells is necessary for repair of the epithelial linings

of organs, including the skin. Because high doses of

5   cycle specific chemotherapeutic drugs, such as ara-C,

effectively kill hematopoietic stem cells and stem cells

of epithelial tissues, patients exposed to such agents

suffer from serious side effects and are frequently at

risk of serious infections.

10   Similar destruction of stem cells occurs upon

exposure to a variety of dosages of radiation, whether

the radiation is used for therapeutic purposes or is the

result of accidental or unavoidable exposure to

radiation, e.g., incident to a clean-up of, or presence

15   at the location of, a nuclear accident at a power plant,

or on a nuclear submarine. Subjects exposed to radiation

also experience the destruction of their hematopoietic

stem cells, and consequent failure or serious damage to

the hematopoietic and immune systems. Radiation also

20   kills dividing epithelial cells with consequent damage to

many epithelial tissues.

While agents, such as the colony stimulating

factors, e.g., M-CSF, CSF-1, GM-CSF, and others, are

presently being employed to stimulate the development of

25   certain hematopoietic cell lineages in subjects exposed

to chemotherapy or radiation, such agents are not

believed to be capable of restoring the hematopoietic

4

system if an insufficient quantity of stem cells is
present in the subject after such exposure.

Thus, there remains a need in the art for other
therapeutic agents capable of protecting stem cells from
5   the damaging effects of chemotherapy or radiation.

Summary of the Invention

The present invention addresses this need in
the art by providing a method of treating a subject
anticipating exposure to an agent capable of killing
10   dividing or cycling stem cells by administering to that
subject an effective amount of a stem cell inhibitory
composition. The stem cells protected by this method may
be hematopoietic stem cells ordinarily present and
dividing in the bone marrow. Alternatively, stem cells
15   may be epithelial, located e.g., in the intestines or
scalp or other areas of the body. The method of this
invention may be desirably employed on humans, although
animal treatment is also encompassed by this method.

The stem cell inhibitory composition useful in
20   this method comprises an effective amount of a
polypeptide or fragment of a protein referred to herein
as Stem Cell Inhibitor Factor (SCIF) and described with
specificity below.

5

In another aspect, the invention provides a
method for protecting and restoring the hematopoietic,
myeloid and immune systems of a patient undergoing
chemotherapy, which includes administering to the patient
5   an effective amount of SCIF. SCIF may be administered
prior to the chemotherapy. Alternatively the SCIF may be
administered during chemotherapy. Another alternative is
administering the SCIF for a period after the
chemotherapeutic regimen. Following chemotherapy, the
10   patient may be treated with therapeutic factors, such as
colony stimulating factors or other lymphokines that
stimulate the stem cells to divide and further stimulate
the production of more mature cells of the hematopoietic
lineages.

15   In still a further aspect, the present
invention involves a method for adjunctively treating any
cancer, including those characterized by solid tumors, by
administering to a patient having cancer an effective
amount of SCIF to protect the hematopoietic stem cells of
20   the bone marrow, thereby allowing greater dosages of
chemotherapeutics or radiation to be employed for
treatment of the cancer.

6

Yet another aspect of the present invention involves the treatment of leukemia comprising treating bone marrow cells having proliferating leukemia cells therein with an effective amount of SCIF to inhibit proliferation of normal stem cells, and treating the bone marrow with a cytotoxic agent to destroy leukemia cells. This method may be enhanced by the follow-up treatment of the bone marrow with other agents that stimulate its proliferation, e.g., lymphokines. This method may be performed in vivo. Alternatively, this method may be performed ex vivo and the resulting marrow purged of leukemia cells by the chemotherapeutic agent. The marrow may then be reinjected into the patient.

In still a further aspect, the method involves treating a subject having any disorder caused by proliferating stem cells. Such a disorder, such as psoriasis, may be treated by administering to the subject an effective amount of SCIF to partially or wholly inhibit proliferation of the stem cell in question.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of preferred embodiments thereof.

7

Detailed Description of the Invention

The present invention provides a method for reversibly protecting stem cells from damage from a cytotoxic agent capable of killing dividing stem cells. The method involves administering to a subject anticipating exposure to such an agent an effective amount of a Stem Cell Inhibitory Factor (SCIF). The method also may involve extending the SCIF treatment throughout the cytotoxic treatment to enhance the stem cell protective effect.

SCIF can reversibly inhibit division of a variety of stem cells in the human body. Specifically SCIF is effective in temporarily inhibiting cell divisi· of hematopoietic stem cells. Additionally, SCIF also acts to inhibit cycling or dividing epithelial stem cells, located throughout the body. Other stem cell populations on which SCIF may exercise a reversibly inhibiting activity include male and female germinal cells. SCIF may then be employed to protect a male or female patient from post-chemotherapeutic germinal aplasia. Additionally, because chemotherapeutic agents commonly induce alopecia and mucositis, SCIF may be use〈 to reversibly protect hair follicles and oro-gastrointestinal epithelial stem cells from adverse sid〈 effects of chemotherapy or radiation.

8

Thus the method of this invention may be
employed in alleviating the undesirable side effects of
chemotherapy on the patient's hematopoietic, myeloid and
immune systems by protecting stem cells from damage from
5   a chemotherapeutic agent, such as a cytotoxic drug or
radiation dosage normally used to destroy cancer cells.
Such an application of SCIF may also serve to protect
epithelial stem cells during chemotherapy. SCIF may be
administered to the patient in a dosage sufficient to
10   inhibit stem cell division for a time sufficient to allow
the action of the chemotherapeutic agent. After the
chemotherapeutic agent has performed its function, the
stem cells inhibited by SCIF will without further
treatment, revert to dividing cells. If it is desired to
15   enhance the reversion of hematopoietic stem cells, the
patient can receive doses of hematopoietic growth factors
or cytokines used to stimulate the growth and development
of hematopoietic cells.

The majority of chemotherapeutic agents used
20   for cancer chemotherapy have a relatively short _in vivo_
half-life, usually less than 24 hours. This inhibitory
effect of SCIF according to the present invention is
maintained for at least the major proportion of the
effective time during which the chemotherapeutic agent is
25   active _in vivo_. For those cytotoxic agents which have

9

prolonged half-lives (e.g., greater than 24 hours), it is
expected that more prolonged treatment with SCIF would be
required. The normal physiological mechanisms within the
subject would limit the effective duration of activity of
5   SCIF in relation to cycling stem cells.

Additionally, the method has utility in
providing a subject protection against other exposure to
radiation, which damages the bone marrow cells of a
subject. An individual may be administered SCIF where
10 unintentional or accidental exposure to dangerous levels
of radiation are anticipated. For example, such
individuals anticipating entering sites of nuclear fall-
out, persons responsible for examining and cleaning
nuclear power plants after a leak of dangerous levels of
15 radiation or nuclear submarines and the like may be
treated by this method to inhibit replicating stem cell
division in case of short term radiation exposure. SCIF
administration during, as well as before radiation
exposure may provide enhanced protection.
20 Additionally, SCIF may be employed in this method as an
adjunctive treatment with chemotherapy to treat any
cancers. Because the bone marrow is the limiting organ
in determining the amount of radiation or dosage of
cytotoxic drug that can be applied to a patient, SCIF may
25 be used to protect the bone marrow hematopoietic cells

10

from radiation or chemotherapy, thereby allowing greater

amounts of radiation or drugs to be applied to treat any

cancer, normally amenable to either radiation or

chemotherapeutic treatment. Since the myelotoxicity of

5   the cytotoxic drugs is also limiting on their dosage

during chemotherapy, the administration of SCIF to the

patient is likely to enable increased drug dosages to be

given to the patient without the serious side effects

that would normally accompany such increased dosages.

10   The method may also be employed to treat solid

tumors by inhibiting during chemotherapeutic treatment,

the division of epithelial stem cells.

SCIF may also be employed in a method for

preparing autologous bone marrow for transplantation.

15   The marrow can be treated _ex_ _vivo_ with an effective

amount of SCIF to inhibit stem cell division and then

purged of cancerous cells by administering an effective

amount of a chemotherapeutic agent or radiation. Marrow

thus treated may be reinjected into the autologous donor.

20   Optionally the patient may then be treated with an agent

known to stimulate hematopoiesis.

SCIF may also be employed in the method of this

invention as an adjunctive therapy in the treatment of

leukemia. For example, where the leukemic cells do not

25   respond to SCIF, the leukemic bone marrow cells may be

11

treated _ex_ _vivo_, with SCIF. The proliferation of normal

stem cells is prevented by administration of SCIF. Thus,

during the time that the proliferating leukemic cells are

treated with a cytotoxic agent, a quantity of normal stem

5   cells are protected from damage. Additionally, a

stimulatory cytokine, such as IL-3 or GM-CSF, may be

administered to induce cycling in the leukemic cells

during drug or radiation treatment while the normal stem

cells are protected with SCIF.

10   For _ex_ _vivo_ use, the bone marrow may be treated

with SCIF to inhibit stem cell division, thereby

protecting stem cells from destruction by the subsequent

application to the marrow or to the source of any other

cancer, of chemotherapy designed to destroy cycling

15   cells, e.g. leukemic cells. The resulting purged marrow

may be reinjected into the patient, wherein the stem

cells will begin to divide normally. To enhance cell

division of the hematopoietic stem cells, the lymphokines

and cytokines above-identified may also be administered.

20   The same process may be accomplished by administering

SCIF to the patient _in_ _vivo_.

The method of this invention may also be

employed to treat disorders related to hyperproliferative

stem cells. For example, psoriasis is a disorder caused

25   by hyperproliferating epithelial cells of the skin and is

12

sometimes treated with cytotoxic drugs. Similarly, a
condition of _in situ_ dysplasia of cervical epithelium is
recognized which will proceed to cervical cancer if left
untreated. Both of these transitional states may be

5   treated prophylactically with SCIF. Other pre-neoplastic
lesions in which stem cell proliferation is involved may
also be amenable to effective amounts of SCIF employed to
inhibit wholly or partially the proliferation of the stem
cells. For these uses, topical or transdermal

10   compositions containing SCIF may be employed, as well as
other parenteral means of administering SCIF.

The SCIF polypeptides may also be used in
another method of this invention. Antibodies, monoclonal
or polyclonal, may be developed by standard techniques to

15   the SCIF polypeptides. These antibodies or SCIF
polypeptides may be labelled with detectable labels of
which many types are known in the art. The labeled SCIF
or anti-SCIF antibodies may then be employed as stem cell
markers to identify and isolate stem cells by

20   administering to a patient directly for diagnostic
purposes. Alternatively these labeled polypeptides or
antibodies may be employed _ex vivo_ to identify stem cells
in a bone marrow preparation, to enable their removal
prior to purging techniques. In the same manner such

13

labeled polypeptides or antibodies may be employed to isolate and identify epithelial, or other stem cells.

The inventors herein have discovered that SCIF, employed in the following methods and examples, is the

5   murine homologue of protein factors previously identified. K. Obaru et al, _J. Biochem._, 99:885-894 (1986) identified the amino acid and DNA sequence of a gene found to be inducible by tumor promoters such as phorbol ester in human tonsillar lymphocytes. The

10   authors predicted that this gene produced a protein which may signal proliferation of T cells. More recently, P. F. Zipfel et al, _J. Immunol._, 142:1582-1590 (1989) reported that the same protein was produced by mitogenic activation of human T cells and suggested that it may

15   function as a lymphokine or cytokine.

The present method thus desirably employs the human SCIF factor in human therapy. This SCIF factor may be the human polypeptide encoded by these prior art sequences. The Obaru/Zipfel SCIF sequence is reported

20   below in Table I. Alternatively, this factor may be obtained as described in Example 1 below. The human analog of SCIF was obtained from a human T cell line using oligonucleotides based on the published LD 78

14

sequence of Obaru et al, cited above. Human SCIF

demonstrates human activity identical to the murine

protein in the SCIF assays described below.

The human SCIF clone of Example 1 has been

5   substantially sequenced. While exhibiting the required

activity, this sequence has been found to differ in both

nucleotide and amino acid sequence from the published

sequence. The alternative human SCIF DNA and amino acid

sequence of Example 1 is reported below in Table II. The

10   sequence within the brackets has not yet been determined.

The sequence that does appear within the brackets on

Table II has been derived from the published LD 78

sequence of Obaru et al and may or may not be identical

with the human SCIF clone. The differences in the

15   nucleotide sequence between the cloned SCIF sequence of

Example 1 and the published Obaru sequence are indicated

in Table II by asterisks above the changed or added amino

acids and below the changed nucleotides. Dashes indicate

non-coding sequences not found in the sequence of Example

20   1. There are 18 nucleotide differences and 4 amino acid

differences between the two SCIF sequences. Additionally

the human clone includes 3 more nucleotides and an

additional amino acid in contrast to the published

sequence.

15

However, also useful in the present invention
as a SCIF molecule are other proteins encoded by other
DNA sequences, not identical to that of the prior art
references. Such DNA sequences are characterized as

5   capable of hybridizing under stringent hybridization
conditions [see, T. Maniatis et al, Molecular Cloning (A
Laboratory Manual), Cold Spring Harbor Laboratory (1982),
pages 387 to 389] to the human SCIF (or Obaru et al) DNA
sequences and coding on expression for polypeptides or

10   proteins capable of demonstrating SCIF activity as
described herein. An example of one such stringent
hybridization condition is hybridization at 4XSSC at
65°C, followed by a washing in 0.1XSSC at 65°C for an
hour.   Alternatively an exemplary stringent

15   hybridization condition is in 50% formamide, 4XSSC at
42°C.

Other human SCIF proteins or polypeptides may
be encoded by other DNA sequences which hybridize to the
sequences for human SCIF (Obaru et al sequence and the

20   sequence obtained from the clone of Example 1) under
relaxed hybridization conditions and which code on
expression for peptides having SCIF biological
properties. Examples of such non-stringent hybridization
conditions are 4XSSC at 50°C or hybridization with 30-40%

25   formamide at 42°C. For example, the molecule identified
previously as human MIP-beta, may be useful in the method

16

of this invention, if capable of displaying stem cell

inhibitory activity as defined hereinbelow by assay.

In a like manner other SCIF polypeptides may be

characterized by amino acid sequences which differ from

5   the Obaru et al sequence or the sequence obtained from

the clone of Example 1 due to allelic variations

(naturally-occurring base changes in the species

population which may or may not result in an amino acid

change). SCIF polypeptides encoded by DNA sequences

10   which differ in codon sequence due to the degeneracies of

the genetic code or allelic variations are also expected

to be useful in the method of this invention. Variations

in the DNA sequence of SCIF which are caused by point

mutations or by induced modifications to enhance the

15   activity, half-life or production of the polypeptides

encoded thereby are also encompassed in the invention and

expected to be useful in the disclosed method.

The method of the present invention may also

employ the murine factor (also known as macrophage

20   inflammatory protein I-alpha, MIP-1 alpha). Murine MIP-

1 beta does not have stem cell inhibitory activity. The

sequence of the molecule here identified as murine SCIF

is reported in Davatelis et al, _J. Exp. Med._, _167_:1939-

1944 (1988) incorporated herein by reference. The stem

25   cell inhibitory function of this molecule was not

17

recognized in that reference. The murine factor may also
be employed in the methods of this invention, provided
that it provokes no antibody generation from the human
immune system.

5   Obviously other species analogs of SCIF may be
employed in various veterinary uses of the above
identified methods.

18

TABLE I

```
AAGGACACGG GCAGCAGACA GTGGTCATGC CTTTCTTGGC                    40

TCTGCTGACA CTCGAGCCCA CATTCCGTCA CCTGCTCAGA ATC                83


ATG CAG GTC TCC ACT GCT GCC CTT GCT GTC CTC CTC TGC           122
Met Gln Val Ser Thr Ala Ala Leu Ala Val Leu Leu Cys
 1           5                    10

ACC ATG GCT CTC TGC AAC CAG TTC TCT GCA TCA CTT GCT           161
Thr Met Ala Leu Cys Asn Gln Phe Ser Ala Ser Leu Ala
        15              20              25

GCT GAC ACG CCG ACC GCC TGC TGC TTC AGC TAC ACC TCC           200
Ala Asp Thr Pro Thr Ala Cys Cys Phe Ser Tyr Thr Ser
            30              35

CGG CAG ATT CCA CAG AAT TTC ATA GCT GAC TAC TTT GAG           239
Arg Gln Ile Pro Gln Asn Phe Ile Ala Asp Tyr Phe Glu
40              45              50

ACG AGC AGC CAG TGC TCC AAG CCG GGT GTC ATC TTC CTA           278
Thr Ser Ser Gln Cys Ser Lys Pro Gly Val Ile Phe Leu
        55              60              65

ACC AAG CGA AGC CGG CAG GTC TGT GCT GAC CCC AGT GAG           317
Thr Lys Arg Ser Arg Gln Val Cys Ala Asp Pro Ser Glu
            70              75

GAG TGG GTC CAG AAA TAT GTC AGC GAC CTA GAG CTG AGT           356
Glu Trp Val Gln Lys Tyr Val Ser Asp Leu Glu Leu Ser
80              85              90

GCC TGA GGGGTCCAGA AGCTTCGAGG CCCAGCGACC                      392
Ala End


TCGGTGGGCC AGTGGGGAGG AGCAGGAGCC TGAGCCTTGG GAAACATGCG 442

TGTGACCTCC ACAGCTACCT CTTCTATGGA CTGGTTGTTG CCAAACAGCC 492
```

19

```
ACACTGRGGG ACTCTTCTTA ACTTAAATTT TAATTTATTT ATTTATACTA 542

TTTAGTTTTT GTAATTTATT TTCGATTTCA CAGTGTGTTT GTGATTGTTT 592

GCTCTGAGAG TTCCCCTGTC CCCTCCCCCT TCCCTCACAC CGCGTCTGGT 642

GACAACCGAG TGGCTGTCAT CAGCCTGTGT AGGCAGTCAT GGCACCAAAG 692

CCACCAGACT GACAAATGTG TATCGGATGC TTTTGTTCAG GGCTGTGATC 742

GGCCTGGGGA AATAATAAAG ACGCTCTTTT AAAAGGTAAA AAAAAAAAAA 792

AAAAAA                                               798
```

20

TABLE II

```
                                    *              **
- - - CTCGAGCCCA CATTCCATCA CCTGCTCCCA ATC ATG CAG GTC   42
                                         Met Gln Val
                                          1

TCC ACT GCT GCC CTT GCC GTC CTC CTC TGC ACC ATG GCT      81
Ser Thr Ala Ala Leu Ala Val Leu Leu Cys Thr Met Ala
     5                   10                  15

            *    ***              *
CTC TGC AAC CAG GTC CTC TCT GCA CCA CTT GCT GCT GAC      120
Leu Cys Asn Gln Val Leu Ser Ala Pro Leu Ala Ala Asp
             .20  *   *         *25

                                            *
ACG CCG ACC GCC TGC TGC TTC AGC TAC ACC TCC CGA CAG      159
Thr Pro Thr Ala Cys Cys Phe Ser Tyr Thr Ser Arg Gln
 30              35                  40

ATT CCA CAG AAT TTC ATA GCT GAC TAC TTT GAG ACG AGC      198
Ile Pro Gln Asn Phe Ile Ala Asp Tyr Phe Glu Thr Ser
         45                  50                  55

                         *
AGC CAG TGC TCC AAG CCC AGT GTC ATC TTC CTA ACC AAG      237
Ser Gln Cys Ser Lys Pro Ser Val Ile Phe Leu Thr Lys
                 60   *           65

*   *
AGA GGC CGG CAG GTC TGT GCT GAC CCC AGT GAG GAG TGG      276
Arg Gly Arg Gln Val Cys Ala Asp Pro Ser Glu Glu Trp
*70                  75                  80

            *       *       *
GTC CAG AAA TAC GTC AGT GAC CTG GAG CTG AGT GCC TGA      315
Val Gln Lys Tyr Val Ser Asp Leu Glu Leu Ser Ala End
             85                  90

[GGGGTCCAGA AGCTTCGAGG CCCAGCGACC TCGGTGGGCC AGTGGGGAGG  365

AGCAGGAGCC TGAGCCTTGG GAAACATGCG TGTGACCTCC ACAGCTACCT   415

CTTCTATGGA CTGGTTGTTG CCAAACAGCC ACACTGRGGG ACTCTTCTTA   465
```

21

```
ACTTAAATTT TAATTTATTT ATTTATACTA TTTAGTTTTT GTAATTTATT    515

TTCGATTTCA CAGTGTGTTT GTGATTGTTT GCTCTGAGAG TTCCCCTGTC    565

CCCTCCCCCT TCCCTCACAC CGCGTCTGGT GACAACC]GA GTGGCTGTCA    615

TCGGCCTGTG TAGGCAGTCA TGGCACCAAA GCCACCAGAC TGACAAATGT    665
     *

GTATCAGATG CTTTTGTTCA GGGCTGTGAT CGGCCTGGGG AAATAATAAA    715
     *

GATGTTCTTT TAAACGGTAA AAA------- --------               738
   *  *        *
```

22

This stem cell inhibitory function of LD78 or
murine MIP alpha identified in the references described
above, has heretofore never been identified as a function
of this protein despite the extensive work of researchers
5   on this factor. See, e.g., Obaru et al and Zipfel et al,
cited above.

The stem cell inhibitory factor acts on cycling
stem cells by reversibly placing them in an undividing
"resting" state. When the stem cells are initially
10   treated in this way, the subsequent application of
chemotherapy or radiation does not kill the stem cells
because the cells are not dividing. Thus, after exposure
to the chemotherapy or radiation, the stem cells may be
"reactivated" to generate dividing progenitor cells, by
15   discontinuing the administration of SCIF.

An additional means of stimulating the resting
stem cells into division may also be the administration
to the subject of other colony stimulating factors, e.g.,
M-CSF, CSF-1, GM-CSF, G-CSF, Meg-CSF or other cytokines,
20   such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9,
IL-11 and erythropoietin following the chemotherapy or
radiation exposure.

23

SCIF polypeptides or active fragments having

stem cell inhibitory activity, may be produced by known

conventional chemical synthesis or recombinant techniques

employing the amino acid and DNA sequences of Table I or

5   Table II, above. For example, SCIF polypeptides may be

produced by culturing a suitable cell or cell line, which

has been transformed with a DNA sequence coding on

expression for a SCIF polypeptide or active fragment

thereof under the control of known regulatory sequences.

10   The resulting protein may be isolated from the cells,

cell lysate, or medium by conventional techniques.

Suitable techniques for such production of recombinant

SCIF are described in, e.g., T. Maniatis et al, Molecular

Cloning-A Laboratory Manual, Cold Spring Harbor

15   Laboratory, Cold Spring Harbor, NY (1982).

Methods for constructing SCIF polypeptides

useful in the method of the present invention by chemical

synthetic means are also known to those of skill in the

art. See, e.g., Merrifield in J.A.C.S, 85: 2149-2154

20   (1963) or Peptide Synthesis" by Bodanszky, et al, second

edition, John Wiley and Sons, 1976.

The recombinant or synthetically-constructed

SCIF polypeptide sequences, by virtue of sharing primary,

secondary, or tertiary structural and conformational

25   characteristics with SCIF polypeptides may possess the

24

same biological characteristic of inhibiting stem cell division in common with the human factor identified above in Table I or Table II.

Modifications in the peptides or DNA sequences 5   encoding SCIF or active fragments thereof may also be made and are believed to be useful also in the method of this invention, where the modified SCIF peptide or fragment thereof shares the desired stem cell inhibitory biological activity. Modifications of interest in the 10   SCIF sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences. Mutagenic techniques for such replacement, insertion or deletion are well known to one skilled in the art. [See, e.g., United States Patent No. 15   4,518,584.]

Specific mutations of the sequences of the SCIF polypeptide may involve the insertion of an asparagine-linked glycosylation site, either Asp-X-Thr or Asp-X-Ser, where X can be any amino acid, into the sequence or a 20   sequence modification at any site of the molecule that is modified by addition of O-linked carbohydrate.

Other analogs and derivatives of the sequence of SCIF which would be expected to retain SCIF activity in whole or in part may also be easily made by one of 25   skill in the art and may be useful in the methods of this invention.

25

Recombinant production of SCIF is presently

preferred. The human cDNA may be isolated and inserted

into suitable cells or cell lines under the control of

appropriate regulatory sequences. Suitable host cells

5   for production of the protein may be mammalian cells,

such as Chinese hamster ovary cells (CHO) or 3T3 cells.

The selection of suitable mammalian host cells and

methods for transformation, culture, amplification,

screening and product production and purification are

10   known in the art. See, e.g., Gething and Sambrook,

Nature, 293:620-625 (1981), or alternatively, Kaufman et

al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et

al, U. S. Patent No. 4,419,446. Other suitable mammalian

cell lines are the monkey COS-1 cell line, and the CV-1

15   cell line.

Similarly useful as host cells suitable for the

production of SCIF for use in the present invention are

bacterial cells. For example, the various strains of E.

coli (e.g., HB101, MC1061 and strains used in the

20   following examples) are well-known as host cells in the

field of biotechnology. Various strains of B. subtilis,

Pseudomonas, other bacilli and the like may also be

employed in this method.

26

Many strains of yeast cells known to those
skilled in the art are also available as host cells for
expression of the polypeptides of the present invention.
Additionally, where desired, insect cells may be utilized
5   as host cells in the method of the present invention.
See, e.g., Miller et al, Genetic Engineering, 8:277-298
(Plenum Press 1986) and references cited therein.

It may also be possible to employ one or more
peptide fragments of SCIF, which retain the stem cell
10   inhibitory activity of intact SCIF molecule, for use in
the present methods for protecting a subject's stem cells
from destruction by exposure to an agent capable of
killing dividing stem cells.

For use in the method for protecting stem cells
15   according to this invention a therapeutically effective
amount of the SCIF protein or a therapeutically effective
fragment thereof may be employed in admixture with a
pharmaceutically acceptable carrier. Where desirable,
this SCIF composition can be systemically administered
20   parenterally. In clinical applications, it may be
desirable to target the SCIF to the blood-forming tissue,
e.g., bone marrow. This targeting can be achieved by
injecting the SCIF, normally by infusion or bolus
intravenous administration. Alternatively, the SCIF can
25   be targeted by varying the pharmaceutical formulation of
the drug, e.g. by linking it to agents which have been

27

shown to target to the bone marrow. This formulation can be administered intravenously. If desirable, the composition may be administered subcutaneously.

When systematically administered, the

5   therapeutic composition for use in this invention is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a pharmaceutically acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is

10   within the skill of the art. For administration in the method for treating hyperproliferating stem cells, the composition containing SCIF may be administered topically or through a transdermal patch to localize its effect on the area of hyperproliferation.

15   The dosage regimen involved in a method for treating the subject anticipating exposure to such cytotoxic agents or for treatment of hyperproliferating stem cells will be determined by the attending physician considering various factors which modify the action of

20   drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 1-1000 micrograms of SCIF protein or fragment thereof per

25   kilogram of body weight.

28

Following the subject's exposure to the

cytotoxic agent or radiation, the therapeutic method of

the present invention may also employ administering to

the subject one or more lymphokines, colony stimulating

5   factors or other cytokines, hematopoietins, interleukins,

growth factors to generally stimulate the growth and

division of the stem cells inhibited by the prior

treatment with SCIF. Such therapeutic agents which

encourage hematopoiesis include IL-1, IL-2, IL-3, IL-4,

10   IL-5, IL-6, IL-7, Meg-CSF, M-CSF, CSF-1, GM-CSF, G-CSF or

erythropoietin. The dosages of these agents may be

employed in the same ranges as the dosages for SCIF

recited above. In a like manner, these dosages would be

adjusted to compensate for variations in the physical

15   condition of the patient, and the amount and type of

chemotherapeutic agent or radiation to which the subject

was exposed. Progress of the reversal of the inhibition

of the stem cells caused by administration of SCIF in the

treated patient can be monitored by conventional methods.

20   In the treatment of leukemia, it may prove

beneficial to administer both SCIF to inhibit normal stem

cell cycling and a stimulator of leukemic cell growth,

such as IL-3 or GM-CSF, simultaneously with the cytotoxic

drug treatment or during radiation. By this protocol, it

25   should be possible to achieve the greatest differences

29

between the cycling statuses of normal and leukemic
cells.

     The following examples illustratively describe
the use of murine SCIF in the in vitro stem cell assay.
5   These examples are for illustration and do not limit the
scope of the present invention.

Example 1 - Obtaining Human SCIF cDNA

     Polyadenylated RNA isolated from the human T
cell line C10-MJ2 was used as a template for synthesis of
10   single stranded complementary DNA by standard procedures
[see, e.g., Maniatis et al., cited above]. The presence
of cDNA's encoding human SCIF were demonstrated by the
polymerase chain reaction (PCR) employing stringent
conditions [R. K. Saiki et al., Science, 230:1350 (1985)]
15   using the following oligonucleotides based on the
sequence of Table I:

     5' AGCTCGAGAT CATGCAGGTC TCCACTG 3'

     5' GCGAATTCCC TCAGGCACTC AGCTCCA 3'.

     Double-stranded SCIF DNA obtained as the
20   product of the PCR and labeled with (alpha ^{32}P) dCTP by
random priming was used to identify SCIF cDNAs in a
previously constructed C10-MJ2 cDNA expression library
[J. F. Moreau et al, Nature, 336:690 (1988)].

30

Conditioned media obtained from COS cells

transfected with full-length SCIF cDNAs in the proper

orientation were analyzed according to the assay

described in Example 3. Sequencing of selected clones,

5   named pXMT2.A1, pXMT2.A3 through pXMT2.A9, is ongoing.

The human SCIF sequence of Table II differs

substantially from the Obaru et al LD 78 sequence of

Table I, from which the oligonucleotides were derived.

There are 18 base changes, 4 amino acid changes as well

10   as three additional bases and an additional amino acid.

Example 2 - In Vitro Stem Cell Assay

For the detection of stem cells in vitro 10^4

murine bone marrow cells from the murine macrophage cell

line, J774.2 [P. Ralph et al, J. Immunol., 114:898-905

15   (1975)] in 4 ml supplemented alpha-modified minimal

essential medium (MEM) containing 25% fetal calf or horse

serum and 0.3% agar were seeded on top of an underlayer

of the same medium containing 0.6% agar, 10% L929 cell

conditioned medium (L929 CM, a source of the growth

20   factor CSF-1) and 10% AF1-19T cell conditioned medium

(AF1-19T CM) (a source of the growth factor GM-CSF and

other uncharacterized stem cell growth factors) in a 6 cm

petri dish. Cultures were incubated at 37°C in a fully

humidified atmosphere of 10% CO_2, 5%, O_2, 85% N_2 for 11

25   days. Colonies can be stained with INT 2-(p-iodophenyl)-

31

3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride hydrate
overnight.

While some colonies are present in the CFU-A
assay having a diameter less than 2 mm, this value was

5   selected as a useful cut-off point after preliminary
experiments were performed using cytosine arabinoside.
Within individual dishes, colonies with a diameter <2 mm
were mainly derived from cells in cycle, whereas colonies
>2 mm were found to be derived from minimally

10   proliferating cells. Only colonies with diameters >2 mm
were scored in these assays.

The assay, also described in I. B. Pragnell et
al, Blood, 72:196-201 (1988), demonstrates that more
mature progenitor cells are unaffected by treatment with

15   SCIF, while cycling stem cells, after treatment with
SCIF, become resistant to the action of cytosine
arabinoside. If the treated stem cells are then washed
with buffered saline to remove the cytotoxic drug and
SCIF the surviving stem cells proliferate in culture

20   normally as illustrated below.

Example 3 - Demonstration of the Effect of SCIF on CFU-A
Bone marrow cells were incubated in paired
tubes containing 5 x 10^6 cells in 1 ml Fischer's medium
supplemented with 20% horse serum. SCIF or alpha-MEM was

25   added to each tube and Fischer's medium was added to

32

control tubes. The mixtures were incubated at 37°C for 5

hours (inhibition assays). For the last 60 minutes of

the incubation 10^{-3}M cytosine arabinoside was added to one

tube and an equal volume of medium to the other tube.

5   Cells were then washed twice before being assayed in the

CFU-A assay as described above. SCIF was found to reduce

the number of stem cells in cycle from an average of

greater than 30% to an average of less than 10%. The

untreated cells were killed by the cytotoxic drug

10   treatment in contrast to the inhibitor treated stem

cells.

Pure preparations of SCIF also reversibly

trigger multipotential stem cells out of cycle when

assayed in vivo using ara-C in the CFU-S assay described

15   in Pragnell et al, cited above.

The purified and sequenced SCIF of this

invention is a cytokine which has the ability to

specifically reduce the proportion of haemopoietic stem

cells in DNA synthesis, thereby protecting them from

20   cytosine arabinoside, a cell-cycle specific cytotoxic

drug. In contrast, SCIF does not affect the

proliferation of more mature progenitors and so appears

to be a specific regulator of the stem cell compartment.

SCIF is active in the 200-500 pM range, as measured in

25   the CFU-A direct addition assays.

33

Example 4 - Expression of Recombinant Human SCIF

To produce SCIF, the cDNA encoding it is
transferred into an appropriate expression vector of
which numerous types are known in the art for mammalian,

5   insect, yeast, fungal and bacterial expression by
standard molecular biology techniques. See, e.g., Y. C.
Yang et al, Cell, 47:3-10 (1986). One such vector is the
COS cell expression vector, pXM, containing the SV40
enhancer, major adenovirus late promoter, DHFR coding

10   sequence, SV40 late message poly A addition site and VaI
gene. This vector may be linearized with the
endonuclease enzyme XhoI and ligated to equimolar amounts
of SCIF cDNA which has been previously modified by the
addition of synthetic oligonucleotides that generate

15   complementary XhoI cohesive ends. Such oligonucleotides
are commercially available [Collaborative Research,
Lexington, MA]. The vector is then introduced into
appropriate host cells by conventional genetic
engineering techniques.

20   a.   Mammalian Cell Expression

To obtain expression of the SCIF
polypeptide for use in the assays described below, the
pXM vector containing the SCIF DNA sequence is
transfected onto COS cells, for example. The conditioned

25   medium for the transfected COS cells contains SCIF

34

biological activity as measured in the assay described in
Example 2.

The mammalian cell expression vectors
described herein may be synthesized by techniques well
5   known to those skilled in this art. The components of
the vectors, e.g. replicons, selection genes, enhancers,
promoters, and the like, may be obtained from natural
sources or synthesized by known procedures. See, Kaufman
et al, J. Mol. Biol., 159:511-521 (1982); and Kaufman,
10   Proc. Natl. Acad. Sci., USA, 82:689-693 (1985).
Exemplary mammalian host cells include particularly
primate cell lines and rodent cell lines, including
transformed cell lines. Normal diploid cells, cell
strains derived from in vitro culture of primary tissue,
15   as well as primary explants, are also suitable.
Candidate cells need not be genotypically deficient in
the selection gene so long as the selection gene is
dominantly acting. For stable integration of the vector
DNA, and for subsequent amplification of the integrated
20   vector DNA, both by conventional methods, CHO cells may
be employed. Alternatively, the vector DNA may include
all or part of the bovine papilloma virus genome [Lusky
et al, Cell, 36:391-401 (1984)] and be carried in cell
lines such as C127 mouse cells as a stable episomal
25   element. Other suitable mammalian cell lines include but
are not limited to, HeLa, COS-1 monkey cells, mouse L-929

35

cells, 3T3 lines derived from Swiss, Balb-c or NIH mice,
BHK or HaK hamster cell lines.

Stable transformants are then screened for
expression of the product by standard immunological or
enzymatic assays. The presence of the DNA encoding the
SCIF polypeptides may be detected by standard procedures
such as Southern blotting. Transient expression of the
DNA encoding the polypeptides during the several days
after introduction of the expression vector DNA into
suitable host cells, such as COS-1 monkey cells, is
measured without selection by activity or immunologic
assay of the proteins in the culture medium.

One skilled in the art can also construct
other mammalian expression vectors comparable to the
pXM/SCIF vector by, e.g., inserting the DNA sequence of
SCIF from the respective plasmids with XhoI and employing
well-known recombinant genetic engineering techniques and
other known vectors, such as pJL3 and pJL4 [Gough et al.,
EMBO J., 4:645-653 (1985)] and pMT2 (starting with pMT2-
VWF, ATCC #67122; see PCT application PCT/US87/00033).
The transformation of these vectors into appropriate host
cells can result in expression of the SCIF polypeptides.

b. Bacterial Expression Systems

Similarly, one skilled in the art could
manipulate the sequence of SCIF by eliminating any
mammalian regulatory sequences flanking the coding

36

sequences and inserting bacterial sequences to create

bacterial vectors for intracellular or extracellular

expression of the SCIF polypeptides of the invention by

bacterial cells. The DNA encoding the factor may be

5   further modified to contain different codons for

bacterial expression as is known in the art. Preferably

the sequence is operatively linked in-frame to a

nucleotide sequence encoding a secretory leader

polypeptide permitting bacterial expression, secretion

10   and processing of the mature variant protein, also as is

known in the art. The compounds expressed in bacterial

host cells may then be recovered, purified, and/or

characterized with respect to physiochemical, biochemical

and/or clinical parameters, all by known methods.

15   c.   Insect or Yeast Cell Expression

Similar manipulations can be performed for

the construction of an insect vector [See, e.g.,

procedures described in published European patent

application 155,476] for expression in insect cells. A

20   yeast vector could also be constructed employing yeast

regulatory sequences for intracellular or extracellular

expression of the proteins of the present invention by

yeast cells. [See, e.g., procedures described in

published PCT application WO 86/00639 and European patent

25   application EP 123,289.]

37

Example 5 - Construction of CHO Cell Lines Expressing
High Levels of SCIF

One method for producing high levels of the
SCIF polypeptides of the invention from mammalian cells
involves the construction of cells containing multiple
copies of the heterologous SCIF gene. The heterologous
gene can be linked to an amplifiable marker, e.g., the
dihydrofolate reductase (DHFR) gene for which cells
containing increased gene copies can be selected for
propagation in increasing concentrations of methotrexate
(MTX) according to the procedures of Kaufman & Sharp, J.
Mol. Biol., (1982) supra. This approach can be employed
with a number of different cell types.

For example, the pXM vector containing a SCIF
gene in operative association with other plasmid
sequences enabling expression thereof and the DHFR
expression plasmid pAdD26SV(A)3 (Kaufman & Sharp, Mol.
Cell Biol., 3(9):1598-1608 (1983) can be co-introduced
into DHFR-deficient CHO cells, DUKX-BII, by calcium
phosphate coprecipitation and transfection.
Alternatively, the SCIF gene may be introduced into pMT2
as previously mentioned and the resultant vector used in
place of pXM/SCIF and pAdD26SV(A)3. DHFR expressing
transformants are selected for growth in alpha media with
dialyzed fetal calf serum, and subsequently selected for
amplification by growth in increasing concentrations of

38

MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as
described in Kaufman et al., _Mol. Cell Biol.,_ 5:1750
(1983). Transformants are cloned, and biologically
active SCIF polypeptide expression is monitored by the
5   assay of Example 2. SCIF polypeptide expression is
expected to increase with increasing levels of MTX
resistance.

Numerous modifications and variations in
practice of this invention are expected to occur to those
10   skilled in the art.

39

CLAIMS

1. A stem cell inhibitory protein substantially free from other human proteinaceous materials and comprising the amino acid sequence of Table II.

2. A pharmaceutical composition useful for inhibiting stem cell division in a human subject exposed to or anticipating exposure to an agent capable of damaging or destroying stem cells undergoing division comprising an effective amount of a human stem cell inhibitory factor in admixture with a suitable carrier.

3. The composition according to claim 2 wherein said stem cell inhibitor is all or a biologically active fragment of the human protein having the same or substantially the same amino acid sequence of Table I, an allelic variant thereof, a polypeptide having a DNA sequence capable of hybridizing to the DNA sequence of Table I and having stem cell inhibiting activity, or a biologically active fragment thereof.

4. The composition according to claim 2 wherein said stem cell inhibitor is all or a biologically active fragment of the protein having the same or substantially the same amino acid sequence of Table II, an allelic variant thereof, a polypeptide having a DNA sequence

40

capable of hybridizing to the DNA sequence of Table II

and having stem cell inhibiting activity, or a

biologically active fragment thereof.

5.   A method for preparing an autologous bone marrow

sample for transplantation comprising treating said

marrow ex vivo with an effective amount of a human stem

cell inhibitory factor substantially free from other

human proteinaceous materials and comprising the amino

acid sequence of Table II to inhibit stem cell division;

and purging said marrow of cancerous cells by

administering an effective amount of a chemotherapeutic

agent or radiation.

6.   A DNA sequence consisting essentially of a DNA coding

on expression for a stem cell inhibitory protein

substantially capable of inhibiting growth of stem cells

in humans, said DNA sequence encoding a peptide sequence

encompassing the mature form of the polypeptide of Table

II and allelic variations thereof also capable of

inhibiting the growth of stem cells in humans.

7.   A vector comprising a DNA sequence consisting

essentially of a DNA coding on expression for a stem cell

inhibitory protein substantially capable of inhibiting

growth of stem cells in humans, said DNA sequence

encoding a peptide sequence encompassing the mature form

41

of the polypeptide of Table II and allelic variations
thereof in association with regulatory control sequences
capable of directing the replication and expression
thereof in host cells.

8. A method for producing a stem cell inhibitory protein
substantially free from other human proteinaceous
materials and comprising the amino acid sequence of Table
II comprising culturing a cell transfected with the DNA
sequence encoding said protein, said DNA under the
control of regulatory control sequences capable of
directing the replication and expression thereof in said
cell.

9. The use of a stem cell inhibitory protein
substantially free from other human proteinaceous
materials and comprising the amino acid sequence of Table
II in a pharmaceutical composition for the treatment of
subjects anticipating exposure to agents which damage
dividing stem cells.

INTERNATIONAL SEARCH REPORT

International Application No **PCT/US 90/05451**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: C 07 K 13/00, A 61 K 35/28, C 12 N 15/19, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC⁵	C 07 K, A 61 K, C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category°	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	The Journal of Immunology, vol. 142, no. 5, 1 March 1989, The American Association of Immunologists, (US), P.F. Zipfel et al.: "Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors", pages 1582-1590 see page 1582, column 2, lines 4-23, 36-42; page 1583, column 1, lines 6-11; page 1585, figure 3B; page 1586, column 1, lines 8-18; discussion cited in the application	1-4,6-9
Y	--	5
Y	EP, A, 0317141 (BECTON DICKINSON AND CO.) 24 May 1989 see claims 1,2,5	5
	--	
	./.	

---

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31st January 1991	2 2. 02. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	miss T. MORTENSEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	J. Biochem., vol. 99, no. 3, 1986, (Tokyo, JP), K. Obaru et al.: "A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter", pages 885-894 see introduction; page 889, figure 3; discussion cited in the application --	1-4,6-9
P,X	Nucleic Acids Research, vol. 18, no. 11, 11 June 1990, S.G. Irving et al.: "Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q", pages 3261-3270 see the whole article	1-4,6-9

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 15/02/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0317141	24-05-89	JP-A- 2002345	08-01-90